Temperature determines the diversity and structure of N_2O-reducing microbial assemblages

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Abstract

Micro-organisms harbouring the *nosZ* gene convert N_2O to N_2 and play a critical role in reducing global N_2O emissions. As higher denitrifier diversity can result in higher denitrification rates, here we aimed to understand the diversity, composition and spatial structure of N_2O-reducing microbial assemblages in forest soils across a large latitudinal and temperature gradient. We sequenced *nosZ* gene amplicons of 126 soil samples from six forests with mean annual soil temperatures (MAST) ranging from 3.7 to 25.3°C and tested predictions of the metabolic theory of ecology (MTE) and metabolic-niche theory (MNT). As predicted, α-diversity of *nosZ* communities increased with increasing MAST, within-site β-diversity decreased and two (pH and soil moisture) of the three niche widths examined were larger with increasing MAST. We calculated β-nearest taxon distance and Raup–Crick

metric to quantify the relative influence of the assembly processes determining *nosZ* assemblage structure. Environmental selection was the primary process driving assemblage structure in all six forests. Homogenizing dispersal was also important at one site, which could be explained by the site's much lower variability in soil chemistry. We used canonical correspondence analysis and multiple regression on matrices to examine relationships between *nosZ* communities and environmental factors, and found that temperature and spatial distance were significant predictors of *nosZ* assemblage structure. Overall our results support both theories (MTE and MNT) tested, showing that higher temperatures are correlated with higher local diversity, wider niche breadths and lower within-site turnover rates.

Keywords: biogeography, diversity, latitudinal diversity gradient, N_2O-reducing community, *nosZ*, temperature gradient

INTRODUCTION

Microbial reduction of nitrous oxide (N_2O), a major greenhouse gas (Ravishankara, Daniel, & Portmann, 2009; Sanford et al., 2012), is the key process allowing soils to serve as N_2O sinks (Jones et al., 2014; Philippot et al., 2013). Bacteria and archaea with N_2O-reductase encoded by the *nosZ* gene (Henry, Bru, Stres, Hallet, & Philippot, 2006; Philippot, Andert, Jones, Bru, & Hallin, 2011) perform the last step in the denitrification pathway (He et al., 2010). As denitrification rates are positively correlated with denitrifier diversity (Jones et al., 2014; Philippot et al., 2013), it is important to understand how environmental factors delimit this vital ecosystem function (Frey, Lee, Melillo, & Six, 2013; Philippot et al., 2009).

The abundance and composition of *nosZ* assemblages are sensitive to temperature (Walker, Egger, & Henry, 2008), moisture (Szukics et al., 2010), vegetation cover, plant diversity (Cavigelli & Robertson, 2000; Philippot et al., 2009), soil organic matter (Morales, Cosart, & Holben, 2010), soil C:N ratio and pH (Rich, Heichen, Bottomley, Cromack, & Myrold, 2003). However, biogeographic-scale patterns of soil *nosZ* communities have not been well investigated, as most of aforementioned studies were largely based on individual locales. Here, we took a different approach and examined *nosZ* assemblage diversity within and across different forests along a temperature gradient at biogeographic scales.

The metabolic theory of ecology (hereafter, “MTE,” Brown, Gillooly, Allen, Savage, & West, 2004) offers a mechanistic explanation for the relationship between diversity and temperature, where mutation and speciation increase exponentially with environmental temperature (Allen, Gillooly, Savage, & Brown, 2006; Gillooly et al., 2006), leading to higher α-diversity at higher temperatures. Our previous work has shown that soil bacterial, fungal and *nifH* communities had temperature–diversity patterns consistent with the MTE (Zhou et al., 2016).

Metabolic-niche theory (hereafter “MNT,” Okie et al., 2015) extends MTE to integrate metabolic principles with niche-based community assembly (in other words, environmental/habitat filtering). MNT posits that niche breadth of free-living microbes will be greater at warmer temperatures due to increased physiological performance, metabolic rates, individual growth rates and decreased generation time. The MNT predicts that this increase in niche breadth of individual taxa also increases α-diversity, without excluding MTE's prediction of increasing diversification with increasing temperature. Increased niche width for an environmental factor (e.g. pH) would also allow species to occupy more of that environmental gradient, thereby increasing the niche overlap within the assemblage and decreasing β-diversity. Thus, MNT predicts a positive relationship between temperature and α-diversity and a negative relationship between temperature and within-site β-diversity (Okie et al., 2015).

Different ecological processes, such as population drift and dispersal, act differently on the assembly and distribution of taxa across space, generating distinct patterns of β-diversity (Stegen, Enquist, & Ferriere, 2009; Vellend, 2010). For example, increased dispersal limitation typically leads to a higher spatial turnover rate. Conversely, a high dispersal rate (“homogenizing dispersal”) generally causes a low compositional turnover (Stegen, Lin, Fredrickson, & Konopka, 2015). Environmental selection may result in remarkably different microbial assemblage structures and turnover rates while drift may also randomly impact microbial community assembly (Vellend, 2010).

Here, we used MiSeq sequencing of *nosZ* gene (clade I) amplicons to examine their diversity in forest soils across a temperature gradient (3.7–25.3°C) ranging from a lowland tropical forest in Panama to a subalpine forest in Colorado. We specifically tested two predictions that derive from MTE and MNT. First, both theories predict that α-diversity is positively correlated with temperature. Second, we test the additional prediction from MNT that β-diversity is negatively correlated with temperature. Additionally, we explored potential ecological and environmental predictors of N_2O-reducing assemblage diversity and composition.

MATERIALS AND METHODS

Sampling and site description

We examined six forests for this study: Niwot Ridge Long Term Ecological Research (LTER) Station, CO (NWT); H.J. Andrews LTER, OR (AND); Harvard Forest LTER, MA (HRF); Coweeta LTER, NC (CWT); Luquillo LTER Puerto Rico (LUQ); and Barro Colorado Island, Panama (BCI). At each forest, we collected soil samples from 21 plots using nested squares 1, 10, 50, 100 and 200 m from a central point (Figure 1). Geographic distance between sites was determined using latitude and longitude (Table S1), and mean annual soil temperature and precipitation data were obtained from hourly temperature

and daily precipitation measures from the nearest weather stations (Table S2). More detailed information can be found at http://macroeco.lternet.edu/?q=node/13.

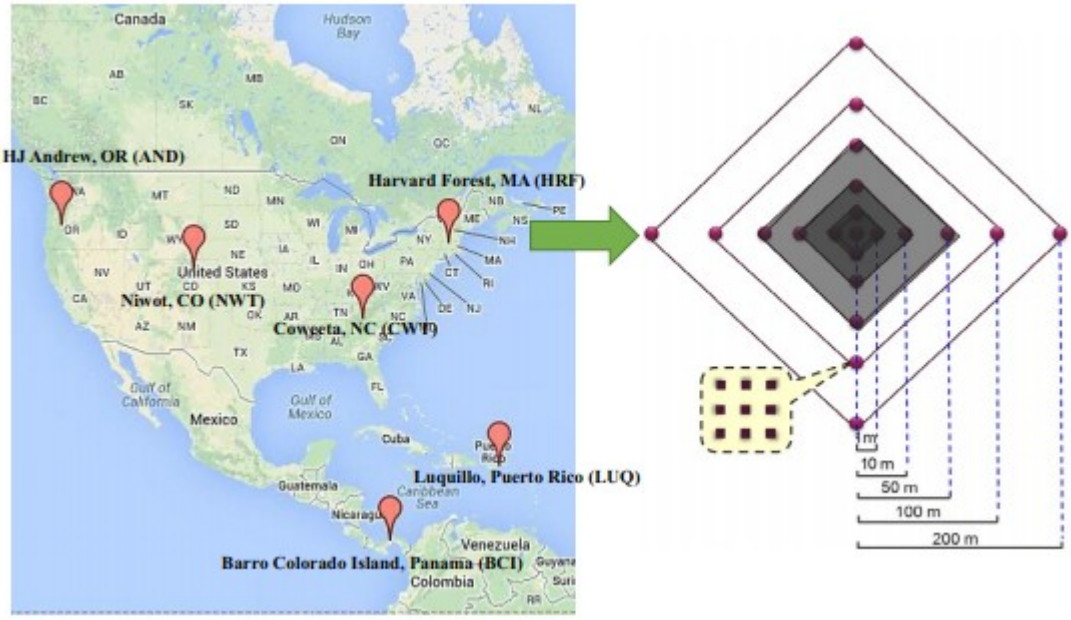

FIGURE 1 Sampling strategy with nested design. Samples were taken from six forest sites in North America. Within each site, 21 subsamples were collected at a distance of 1, 10, 50, 100 and 250 m following a nested sampling strategy. In addition, for each subsample, nine soil cores were collected and composited to reduce soil heterogeneity

Taxa

Here, we examined the *nosZ* clade I, represented by the commonly dominant soil bacterial phylum, *Proteobacteria* (Jones et al., 2014; Philippot et al., 2013). Recently, the *nosZ* clade II was described (Orellana et al., 2014) and showed a strong N_2O sink capability (Domeignoz-Horta et al., 2015), but we only examined the *nosZ* clade I so that our results could be compared to other previous studies (e.g. (Stres, Mahne, Avgustin, & Tiedje, 2004; Walker et al., 2008; Philippot et al., 2013; Jones et al., 2014)) that used the *nosZ* clade I.

Soil property analysis

We measured soil moisture by drying 1.5 g soil in a 65°C oven and calculating SM% = 100*(1.5 - dry mass)/1.5. We measured soil pH by mixing a 1:2.5 (weight: volume) slurry of soil:water, allowing the mix to settle for an hour, and reading pH of the supernatant. Soil chemistry was measured by the Soil, Water and Forage Analytical Laboratory at Oklahoma State University (Stillwater, OK). Carbon (C) and nitrogen (N) contents were measured by a LECO TruSpec Carbon and Nitrogen Analyzer (LECO

Corporation, St. Joseph, MI). Soil $NH_4^{+¿¿}$ and $NO_3^{-¿¿}$ contents were extracted from each soil sample with 1M KCl and measured by an FIA Lachat Quickchem 8500 Series 2 instrument (Lachat, Loveland, CO). Soil property data are shown in Table S1.

Soil DNA extraction

We extracted DNA from soils by freeze-grinding mechanical lysis (Zhou, Bruns, & Tiedje, 1996), and purified using a low melting agarose gel, followed by phenol extraction. DNA quality was assessed based on absorbance ratios of 260/280 nm (~1.80) and 260/230 nm (>1.7) using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and final DNA concentration was quantified by a fluorescent method (Quant-iT™ PicoGreen, Invitrogen MP, Eugene, OR, USA), using a FLUOstar Optima fluorescent plate reader (BMG Labtech, Jena, Germany).

Primer design for the *nosZ* clade I and coverage analysis

The primer design for *nosZ* clade I sequences was performed by the RDP Functional Gene pipeline (http://fungene.cme.msu.edu/FunGenePipeline/). Reference sequences and associated alignment were downloaded from the pipeline, and such an alignment was generated by directly mapping the nucleotides to the protein alignment by HMMER 2.0. Candidate priming regions were selected based on entropies of aligned nucleotide positions calculated using a PYTHON script, and any base substitution (A, T, G and C) contributed to degeneracy of that alignment position was considered if its frequency was at least 10%. Tm calculations were carried out using OLIGOCALC (http://biotools.nubic.northwestern.edu/OligoCalc.html). Finally, the best primer pair was selected: forward primer 5′-STTYMTSGACAGCCARR-3′ and reverse primer 5′-VCGRTCYTTVGAGAAYT-3′, which target an approximate 172-bp sequence of *nosZ* genes. Primer coverage and specificity were checked with SEQFILTER (Fish et al., 2013), and *nosZ* reference database was provided by Dr. Christopher Jones (Swedish University of Agricultural Sciences). The sequence maximum mismatch of 2 was used to access the coverage of all *nosZ* genes in the database, and for clade I *nosZ* genes, the coverage was 85% (224 of 263), while our primers did not have significant hits with clade II *nosZ* genes.

PCR amplification and purification of *nosZ* amplicons

A two-step PCR method was used in this study with Accuprime™ Taq DNA Polymerase High Fidelity kit (Life Technologies, Grand Island, NY), which was expected to increase amplification efficiency and reduce amplification biases. The first step PCR was carried out in 25 µL reaction mixture containing 2.5 µl 10 × PCR buffer II (including dNTPs), 0.25 U high-fidelity AccuPrimeTM Taq DNA polymerase (Life Technologies), 0.04 µM of both forward and reverse target-only primers (mentioned above), and 30 ng of soil DNA. PCR amplification conditions were as follows: one cycle at 95°C for

3 min followed by 15 cycles of 95°C for 30 s, 59°C for 45 s and 68°C for 45 s; and a final extension step at 68°C for 7 min. Then, the triplicate products of first step PCR were combined, purified with Agencourt® AMPure XP kit (Beckman Coulter, Inc.) and eluted in 50 µl water; 10 µl elution was then used as the templates for the second step PCR. The second step PCR primers were a combination of the first primer pair and Illumina MiSeq adaptors with an additional 12-mer tag for the reverse primer, allowing 126 samples to be sequenced in one run. The amplification conditions were as follows: one cycle at 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 49°C for 45 s and 68°C for 45 s; and a final extension step at 68°C for 7 min. Triplicate reactions were prepared for each sample and pooled prior to purification. The final PCR products were visualized on 1% agarose gels after DNA gel electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

MiSeq sequencing of *nosZ* gene amplicons and sequence data preprocessing

Purified PCR products of *nosZ* were quantified by PicoGreen using a FLUOstar Optima (BMG Labtech, Jena, Germany) and then were pooled into a mixture of *nosZ* amplicons with an equimolar amount for each sample. The pooled *nosZ* amplicons were sequenced by an Illumina MiSeq (San Diego, CA, USA). Quality filtering and preprocessing (e.g. de-multiplexing, primer trimming and assembling) of MiSeq reads were conducted using the RDP pipeline at Michigan State University (http://fungene.cme.msu.edu/FunGenePipeline/). RDP FrameBot was used to detect and correct frameshift errors caused by insertions and deletions, and then, the sequence data were resampled at the same number of 15,476 sequences for each sample by our pipelines (http://zhoulab5.rccc.ou.edu:8080/root). Resampled sequences were clustered into operational taxonomic units (OTUs) by UPARSE (Edgar, 2013) at the cut-off of 94% identity of DNA sequences (Konstantinidis & Tiedje, 2005). A *search global* command in Usearch was used to calculate the global identity as the reference of *nosZ* genes from cultured organisms from NCBI, and an 80% identity cut-off was used. Raw sequence data have been submitted to NCBI Sequence Read Archives, accessible through Bioproject PRJNA286014.

We generated maximum likelihood phylogenetic trees at the OTU and genus levels using MEGA 5 with 500 bootstrap replicates (Kumar, Nei, Dudley, & Tamura, 2008). We added Ln-transformed genus abundances to the genus-level phylogenetic tree generated from the representative sequences using the iTOL webserver (Letunic & Bork, 2011).

Statistical analyses

We performed all analyses using an in-house pipeline (http://ieg2.ou.edu/NimbleGen/) and R (R Foundation for Statistical Computing, Vienna, Austria) using the R packages: VEGAN (Oksanen et al., 2013), PICANTE (Kembel et al., 2010), ECODIST (Goslee & Urban, 2007) and HMISC (Harrell & Dupont, 2012).

At the site and plot levels, we calculated OTU richness, Shannon's Index, Simpson diversity and Pielou's evenness as well as Chao1 richness based on the OTU table at a depth of 15,476 sequences per sample. To test for the predicted positive relationship between diversity and temperature, we used linear regression of the natural logarithm of indices of α-diversity vs. 1/KT (where K is the Boltzmann constant and T is MAST in Kelvin) (Brown et al., 2004) as well as linear regression between α-diversity indexes and MAST (Okie et al., 2015). All OTUs were classified to phylotypes down to genera when possible, and a standard linear regression was used to evaluate the relationships between temperature and taxon abundance.

To measure β-diversity, we calculated the exponent *z*, the slope of the taxon area relationship (hereafter "TAR"), for each site by generating the best fit power law regression of $\log_{10}$ (OTU richness) on $\log_{10}$ (area) (Rosenzweig, 1995). To test the MNT prediction that within-site β-diversity will have a negative relationship with temperature, we used linear regression of the TAR slopes (*z* values) on MAST. We also used linear regression to test for relationships between *z* values and other environmental variables, including elevation, precipitation and concentrations of soil nitrate and ammonium.

To test the MNT prediction that niche breadth will be positively correlated with temperature, we calculated each taxon's soil pH, TN and moisture niche width using the abundance-weighted standard deviation of each taxon's distribution with respect to soil pH, TN and moisture (Domeignoz-Horta et al., 2015; Okie et al., 2015). We then calculated the abundance-weighted mean pH, TN and moisture niche widths for all taxa that occur at each site. We used linear regression to test the predicted positive relationship between temperature and these mean niche widths.

To infer the relative influence of environmental selection (i.e. filtering), homogenizing dispersal, dispersal limitation and ecological drift on *nosZ* assemblage structure, we used a phylogenetic β-diversity framework. We calculated observed β-mean nearest taxon distance (βMNTD) using "COMDISTNT" in the R package PICANTE (Webb, Ackerly, & Kembel, 2008). We also calculated the abundance-weighted β-nearest taxon distance (βNTI), which is the number of standard deviation units difference between the observed βMNTD and the mean of the null distribution (Stegen et al., 2013). Values of $|\beta NTI| > 2$ indicate that the observed assemblage is showing significantly higher or lower phylogenetic turnover than expected from purely stochastic and random assembly, implicating niche-based environmental selection. To estimate the importance of ecological drift, we calculated the Raup–Crick metric (RC_{Bray}), which is based on a null model test of Bray–Curtis taxonomic β-diversity index and was calculated using the R script provided by Chase, Kraft, Smith, Vellend, and Inouye (2011). When niche-based environmental selection is not implicated (i.e. $|\beta NTI| < 2$), $RC_{bray} > 0.95$ suggests significant (when $\alpha = 0.05$) dispersal limitation, as samples are more phylogenetically different than expected. Similarly, $RC_{Bray} < -0.95$ suggests significant homogenizing dispersal, as samples are more

phylogenetically similar than expected. Where RC_{Bray} is between −0.95 and 0.95, assemblage structure is not significantly different from the null model of ecological drift (Stegen et al., 2015).

To examine for differences between the six forests in soil properties and *nosZ* assemblage composition, we used detrended correspondence analysis (DCA). We used permutational multivariate analysis (PERMANOVA, ADONIS function in VEGAN) to test for differences in assemblage structure among the six sites. We used canonical correspondence analysis (CCA, VEGAN) and multiple regression on matrices (MRM, ECODIST package, (Goslee & Urban, 2007) to test the relative importance of other abiotic and spatial factors on variation in composition among *nosZ* communities. We included site-level environmental factors (MAST, elevation, precipitation), plot-level soil characteristics (total C, total N, NH_4-N, NO_3-N, pH and moisture) and spatial factors (latitude, PCNM vectors), but only the top ten variables that significantly explained the most variation in *nosZ* communities were shown in the CCA plot. Principle coordinates of neighbour matrices (PCNM, PCNM function in VEGAN package; Dray, Legendre, & Peres-Neto, 2006) are spatial eigenvectors that account for spatial autocorrelation at different scales. We used Bray–Curtis dissimilarity for the *nosZ* assemblages. Environmental distances were the Euclidean distance between log-transformed variables.

RESULTS

We found a total of 1,949,976 high-quality sequences after preprocessing of raw reads, and these sequences were clustered into 2,714 OTUs at a 94% identity threshold (Konstantinidis & Tiedje, 2005) with UPARSE after rarefying to 15,476 reads per sample. We detected 46 genera from four known classes (α-, β- and γ-*Proteobacteria* and *Flavobacteria*) and one unknown group (Table S3). Of the 22 genera with more than 1,000 sequences, the most abundant were *Bradyrhizobium* (47.5% of all sequences), *Azospirillum* (28.5%), *Hyphomonas* (10.6%), *Rhodopseudomonas* (2.8%) and *Oligotropha* (1.40%).

The south-eastern deciduous forest (CWT) had the highest OTU richness (1,843); the western coniferous forests had the lowest (NWT: 1,155; AND: 1,190); the two tropical sites (BCI: 1,683; LUQ: 1,634) and the north-eastern deciduous forest (HRF: 1,611) were intermediate (Table 1). The rarefaction curves of Chao1 values were nearly asymptotic (Figure S1), indicating the sequencing effort sufficiently sampled the N_2O-reducing assemblages.

Table 1. Richness and diversity indices of *nosZ* genes and climate variables in all forest soil samples

Site[a]	NWT	AND	HRF	CWT	LUQ	BCI
OTU number	1,155	1,190	1,611	1,843	1,634	1,683
Shannon Index (H')	3.30 ± 0.56 b	1.61 ± 1.12 c	3.70 ± 0.77 b	4.40 ± 0.10 a	4.22 ± 0.50 a	4.43 ± 0.33 a
Simpson Index (D')	13.74 ± 9.11 b	3.82 ± 6.22 c	12.07 ± 4.98 b	15.51 ± 1.60 b	31.42 ± 12.04 a	35.17 ± 18.30 a
Pielou evenness (J')	0.62 ± 0.07 b	0.30 ± 0.17 c	0.62 ± 0.06 b	0.66 ± 0.012 ab	0.70 ± 0.05 a	0.71 ± 0.04 a
Chao1	5.53 ± 0.36 c	5.67 ± 0.50 c	6.23 ± 0.77 b	6.98 ± 0.07 a	6.25 ± 0.33 b	6.52 ± 0.10 b
MAST (°C)	3.73	6.94	8.83	12.12	22.6	25.3
Latitude (°)	40.04	44.23	42.54	35.05	18.32	9.16
Longitude (°)	105.56	122.15	72.18	83.43	65.82	79.85

Values represent $M \pm SD$ ($n = 21$). Data in the same row followed by the same letter are not significantly different at the $p < .05$ level.

The symbols for these sites are as follows: NWT: Niwot Ridge, Colorado; AND: H.J. Andrews Experimental Forest, Oregon; HRF: Harvard Forest, Massachusetts; CWT: Coweeta Hydrological Laboratory, North Carolina; LUQ: Luquillo, El Yunque National Forest, Puerto Rico; BCI, Barro Colorado Island, Panama.

As predicted by MTE and MNT, the OTU richness of *nosZ* communities generally increased with increasing MAST. The slope of the Arrhenius relationship (natural logarithm of OTU richness and α-diversity indices vs. 1/KT) was negative, with a plot-level slope of −0.25 (Ln (OTU richness) = −0.247 × 1/KT + 15.9, $p < .01$, $R^2 = .19$, $n = 126$, Figure 2a). The site-level regression had marginal significance and a less steep slope (Ln (OTU richness) = −0.106 × 1/KT + 11.6, $p = .06$, $R^2 = .44$, $n = 6$, Figure 2b). The negative slopes of the Arrhenius relationship indicated that richness of *nosZ* communities increased exponentially with MAST, with an activation energy of 0.106 and 0.247 eV at the site and plot levels, respectively. Both Chao1 and Simpson diversity increased with temperature (Ln (Chao1) = −0.232 × 1/KT + 15.597 Chao1: $R^2 = .18$, $p < .01$, Figure 2c; Ln (Simpson diversity) = −0.556 × 1/KT + 25.103 Simpson diversity: $R^2 = .40$, $p < .01$, Figure 2d). Positive linear relationships between richness/α-diversity indices of *nosZ* communities and MAST were also generally significant (Figure S2).

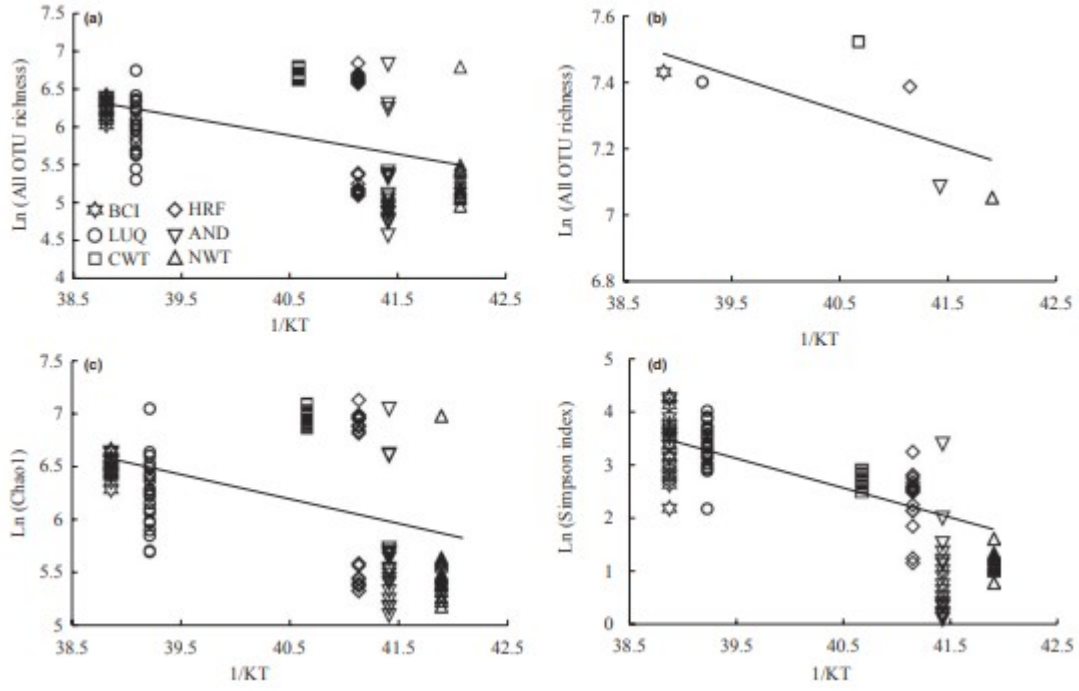

FIGURE 2 The relationships between richness/α-diversity indices of *nosZ* genes and temperature to test the metabolic theory of ecology of N_2O-reducing communities in forest soils. The correlation between the natural logarithm of α-diversity/richness of *nosZ* genes and mean annual soil temperature (MAST) was calculated by the Pearson correlation approach, and temperature was expressed as the inverse of MAST in degree of Kelvin (1/KT). (a) OTU richness at the plot level (n = 126, R^2 = .19, p < .01); (b) OTU richness at the site level (n = 6, R^2 = .44, p = .06); (c) Chao1 at the plot level (n = 126, R^2 = .18, p < .01); and (d) Simpson index at the plot level (n = 126, R^2 = .40, p < .01)

Consistent with the predictions of MNT, β-diversity showed a negative relationship with temperature. The z values of the site-level TARs (Figure 3a) decreased as temperature increased (Figure 3b), indicating lower spatial turnover of taxa at higher temperatures ($z = -0.0044 \times T + 0.1654$, p = .03, R^2 = .55). While the overall relationship between z values and MAST was significant, the lowest value of z was observed at CWT, which had an intermediate MAST. Removing CWT as an outlier gave a similar slope and greatly improved the explanatory power of the model ($z = -0.0047 \times T + 0.1826$, p < .001, R^2 = .96; Figure 3b). However, no significant relationships were observed between the z values and other environmental factors (e.g. latitude, elevation, precipitation, soil nitrate or ammonium and plant richness; see Table S4).

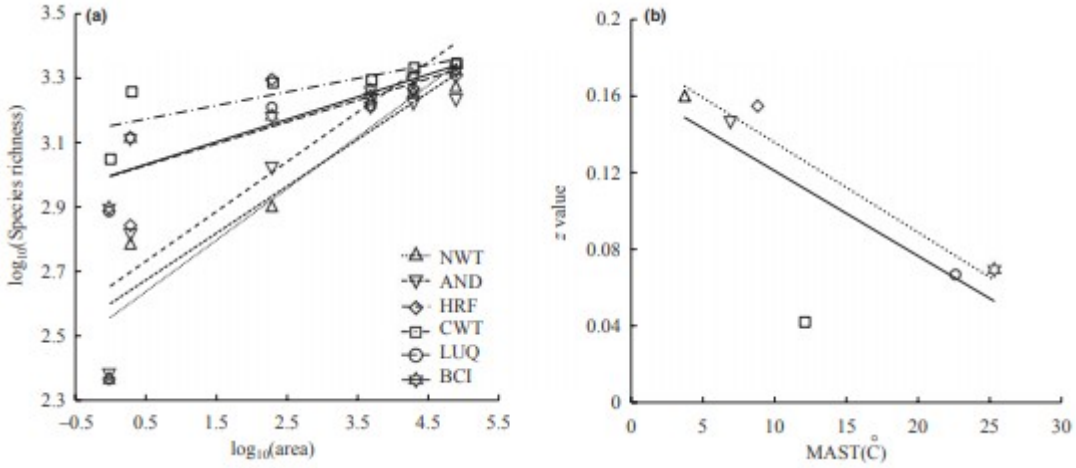

FIGURE 3 The taxon–area relationship (TAR) of *nosZ* genes within each individual site (a) and across the temperature gradient (b). (a) TAR was significant in each individual site (NWT: $z = 0.160$, $R^2 = .87$, $p = .002$; AND: $z = 0.146$, $R^2 = .83$, $p = .004$; HRF: $z = 0.154$, $R^2 = .73$, $p = .017$; CWT: $z = 0.042$, $R^2 = .64$, $p = .036$; LUQ: $z = 0.067$, $R^2 = .79$, $p = .009$; BCI: $z = 0.070$, $R^2 = .84$, $p = .004$); (b) relationship between z values and MAST was performed by the Pearson correlation (all six sites, $n = 6$, $R^2 = .55$, $p = .034$; excluding CWT: $n = 5$, $R^2 = .96$, $p = .003$)

As predicted by MNT, abundance-weighted mean niche width increased with MAST (Figure **4**). Niche width increased significantly with temperature for pH (pH niche width = 0.0053 × MAST + 0.031, $n = 6$, $p < .001$, $R^2 = .91$) and for soil moisture (moisture niche width = 0.0029 × MAST + 0.057, $n = 6$, $p = .019$, $R^2 = .63$). Niche with for TN showed the predicted sign, but the regression was not significant (TN niche width = 0.0042 × MAST + 0.056, $n = 6$, $p = .076$, $R^2 = .40$).

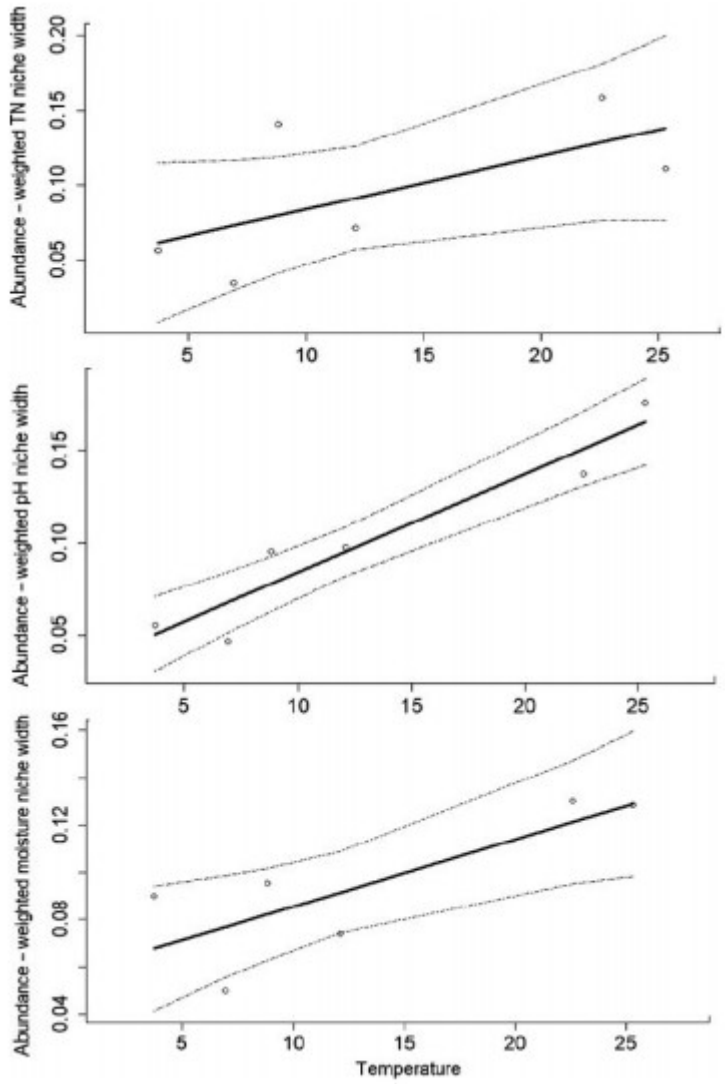

FIGURE 4 Relationships between abundance-weighted mean niche width of taxa in each assemblage (a. total nitrogen, b. pH and c. moisture) and temperature. A taxon's niche width was quantified as the abundance-weighted standard deviation of its distribution along each soil variable with temperature held constant. For each site, the abundance-weighted niche width of taxa in each sample was calculated and averaged across all samples. The mean abundance-weighted niche width across all samples in a site is shown by the circle symbols. Linear regressions between abundance-weighted average niche width and temperature shown are indicated by bold lines and dashed lines indicate 95% prediction intervals

Of the four ecological processes structuring phylogenetic β-diversity of *nosZ* assemblages, we found that environmental selection was primary in five of the six sites, explaining from 61.4% to 80.5% of *nosZ* assemblage turnover (Figure 5). At CWT, homogenizing dispersal (46.7%) was almost as important as environmental selection (47.1%), while no other process contributed more than 6.2%. At the four extra-tropical sites, dispersal limitation contributed

only 0.95% to 2.4%, but contributed much more to the tropical sites (LUQ = 19.0%, BCI = 7.6%). CWT was much more tightly clustered than other sites, indicating more homogenous soils (Figure S3). The PERMANOVA analysis showed that the assemblage composition of all six sites was significantly different from each other (Table S5) with some minimal overlap between some of the paired comparisons when visualized in DCA space, such as CWT, BCI and HRF, and HRF and AND (Figure S4).

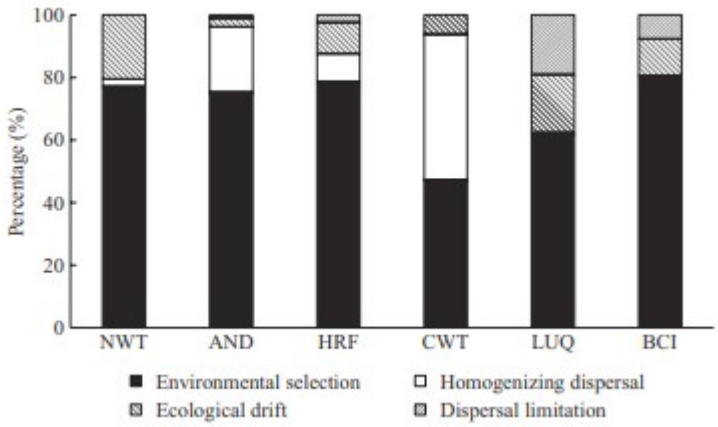

FIGURE 5 Quantification of the influence of ecological processes on the *nosZ* community assembly. Environmental selection results from the influence exerted by the abiotic and biotic environment, inevitably leading to a change in the composition of organisms. Homogenizing dispersal refers to high dispersal rates between communities causing low compositional turnover, whereas dispersal limitation refers to low dispersal rates. Ecological drift refers to a mixture of stochastic organismal movements and birth–death events

Both CCA (Figure 6) and MRM (Table 2) models showed significant relationships between *nosZ* assemblage structure and environmental variables (CCA: $R^2 = .68$, $p = .001$; MRM: $R^2 = .28$, $p = .001$). CCA Axis 1 was significantly ($R^2 = .59$, $p < .01$) correlated with temperature. Notably, CCA1 values for CWT were more tightly clustered than for the other sites, only 0.08 units compared to 0.20–0.92 for the other five sites. Thus, CWT plots appeared to be more similar to each other than plots in the other sites. Geographic distance, MAST, elevation, precipitation and soil properties all significantly affected the composition of *nosZ* assemblages at the biogeographic scale (Figure 6). MRM analysis showed four variables with significant partial regression coefficients, MAST being the best predictor of *nosZ* assemblage structure (Table 2, partial regression coefficients: MAST, $b = 0.06$; geographic distance, $b = 0.05$; NO_3-N, $b = 0.04$; and latitude, $b = -0.03$).

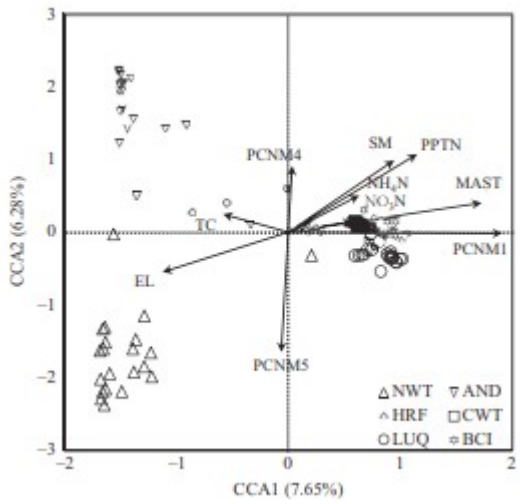

FIGURE 6 Canonical correlation analysis (CCA) of all detected *nosZ* genes in 126 soil samples of six forest sites. PCNM: principle coordinates of neighbour matrices vectors for geographic distance; TC: total carbon; EL: elevation; SM: soil moisture; PPTN: precipitation; MAST: mean annual soil temperature

Table 2. The partial regression coefficients (*b*) and *p* values of the multiple regression on matrices (MRM) analysis of relative contributions of geographic distance and environmental factors

	Coefficient (b)	p value
Ln(distance)	0.0539	.001
Ln (TC)		
Ln (TN)		
Ln (NH_4-N)		
Ln (NO_3-N)	0.0368	.004
pH		
Ln (soil moisture)		
Ln (elevation)		
Ln (precipitation)		
Latitude	−0.0295	.001
MAST	0.0567	.001

The variation (R^2) in Ln (assemblage distance) explained by the remaining variables in the final model was $R^2 = .284$ ($p = .001$).

DISCUSSION

Using soils from six North American forests, we show for the first time that clade I *nosZ* diversity is correlated with temperature at biogeographic scales, which is consistent with MTE predictions (Brown et al., 2004). Additionally, our results are consistent with two key predictions of the MNT: within-site β-diversity is lower and niches are wider at higher temperatures (Okie et al., 2015). As would be expected by these impacts, temperature is a major driver of *nosZ* assemblage structure.

MTE predicts that species richness in free-living organisms increases exponentially with environmental temperature as speciation rate increases with temperature, especially so at regional scales (Brown et al., 2004). As microbial metabolic and growth rates exhibit comparable temperature dependencies to plants and animals (Okie, 2012), species richness of microbial communities should also be temperature-dependent (Stegen et al., 2009). In this study, MAST was marginally predictive of variation in *nosZ* richness at the site scale (slope of −0.11) and significantly predictive at the plot scale (slope of −0.25). However, such values are smaller than the predicted slope of −0.65 for heterotrophic macro-organisms (Allen, Brown, & Gillooly, 2002; Brown et al., 2004), suggesting that micro-organisms and macro-organisms may require different co-evolutionary models (Stegen et al., 2009) or at least that the operational taxonomic unit we employed is not comparable to the species level studied in plant and animal macroecology.

Building from MTE, MNT also predicts the positive relationship between temperature and species richness we observed but offers two additional predictions. As temperature drives metabolic rates of microbes, higher temperatures should increase taxon niche widths which in turn means that species can be more widespread, reducing within-site β-diversity (Okie et al., 2015). Our results are consistent with both of these predictions.

As predicted by the MNT, warmer sites had lower β-diversity. Using the *z* values (i.e. the power law exponent) of the taxon–area relationship as a measure of β-diversity, where a steeper slope indicates higher β-diversity, we found a significant negative relationship between site temperature and β-diversity. The *z* values ranged from 0.16 in a subalpine spruce forest to 0.07 for tropical forests, with the notable outlier in a mixed deciduous forest (0.04) showing the flattest TAR. The *z* values we observed for the *nosZ* assemblages are similar to values of other micro-organisms collected in the same plots [e.g. bacteria from 0.18 to 0.25, fungi from 0.17 to 0.23 and diazotrophic (*nifH* communities) from 0.43 to 0.47 (Zhou et al., 2016)]. There were no significant relationships between *z* values and the other environmental variables tested, highlighting the importance of temperature on the β-diversity of these *nosZ* assemblages.

As predicted by the MNT, abundance-weighted niche width for two soil variables significantly increased with temperature across biogeographic scales, as has also been observed at local scales (Okie et al., 2015). The strongest increase in niche width with temperature was the niche width for pH, which is a major driver of soil microbial diversity and assemblage structure (Fierer & Jackson, 2006). As the intracellular pH of most micro-organisms is generally neutral ± 1 (Booth, 1985; Madigan, Martinko, & Parker, 1997), significant deviation in soil pH should impose strong selective stress on soil micro-organisms.

Our results are thus consistent with a prediction of MTE and three additional predictions of MNT. While increased diversification rates may drive a large-

scale pattern of increased diversity, as predicted by MTE, our results also corroborate MNT's predictions that the metabolic effects of temperature should lead, all-else-being-equal, to environmental niche breadths being wider at higher temperatures, which in turn should lead to higher α-diversity and lower β-diversity at higher temperatures in communities experiencing nonrandom selection and assembly processes. The relevance of MNT to soil forest communities is further bolstered by the observed primacy of environmental selection in shaping the phylogenetic β-diversity of all six forests. All forests other than CWT had at least 60% of phylogenetic β-diversity attributable to environmental selection.

Our findings are consistent with other more local studies and laboratory experiments. Temperature directly (i.e. warming) and indirectly (MAST) impacted the composition of *nosZ* genes in arctic tundra soils (Walker et al., 2008). When a single soil type is incubated at different temperatures, different temperatures appear to select for distinct denitrifying communities (Braker, Schwarz, & Conrad, 2010); denitrifying assemblages in marine sediments appear to be adapted to local temperature conditions (Canion et al., 2014).

While temperature appears to be the major driver, other factors affect within- and among-site patterns of richness and assemblage structure. In addition to differences in temperature, differences in soil chemistry, precipitation as well as purely spatial differences are also driving differences among our six sites. While secondary to temperature in our study, spatial distance between sites appears to explain differences in assemblage structure even after accounting for the effects of measured abiotic variables. We are uncertain whether this is simply due to the large spatial distances between those sites or whether they are spatially autocorrelated with biotic and/or abiotic factors. Large-scale spatial patterns of soil properties have been shown to significantly affect denitrification activity and relative abundances of denitrification genes (Philippot et al., 2009). Environmental filtering thus appears to play a pivotal role in shaping *nosZ* communities in forest soils (Morales, Jha, & Saggar, 2015).

Previous studies have argued that soil pH was a major driver shaping soil microbial assemblages (Fierer & Jackson, 2006) and *nosZ* clade II assemblage (Domeignoz-Horta et al., 2015; Samad et al., 2016). While we did not detect a significant effect of pH on assemblage structure here, this may be due to the range of pH in our study being relatively narrow (3.4–6.6) and clade I *nosZ* assemblages being less sensitive to pH changes than clade II (Domeignoz-Horta et al., 2015; Samad et al., 2016). Nevertheless, we showed a relationship between temperature and pH niche width of clade I *nosZ*, suggesting that pH plays a role in determining the fine-scale distribution of species among samples within forest sites.

Coweeta had the highest OTU richness and the flattest TAR slope at an intermediate MAST. Removing Coweeta as an outlier typically did not change

the nature of the regression models but greatly improved correlation coefficients. The flattest TAR slope indicates that CWT had the lowest taxonomic β-diversity, which was mirrored by relative importance of homogenizing dispersal in governing phylogenetic β-diversity in this forest. Because CWT had, by far, the most homogeneous soils (Figure 6) without having reduced niche breadths, taxa could be more widespread throughout the forest, reducing β-diversity.

Consistent with the predictions of MTE and MNT, clade I *nosZ* assemblages, on average, are more diverse at higher temperatures. Consistent with MNT, clade I *nosZ* assemblages show lower β-diversity and larger niche widths at higher temperatures. Temperature significantly affects the composition of clade I *nosZ* assemblages, and there was significant assemblage structure related to several environmental factors. Additionally, patterns in phylogenetic β-diversity indicate that environmental selection is the major ecological process structuring *nosZ* assemblages. Taken as a whole, our results suggest that temperature and geographic distance are the major drivers shaping N_2O-reducing communities.

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DATA ACCESSIBILITY

Data were submitted to NCBI Sequence Read Archives and available from NCBI Bioproject PRJNA286014.

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